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# Accounting for systematic errors in bioluminescence imaging to improve quantitative accuracy

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## ABSTRACT

Bioluminescence imaging (BLI) is a widely used pre-clinical imaging technique, but there are a number of limitations to its quantitative accuracy. This work uses an animal model to demonstrate some significant limitations of BLI and presents processing methods and algorithms which overcome these limitations, increasing the quantitative accuracy of the technique. The position of the imaging subject and source depth are both shown to affect the measured luminescence intensity. Free Space Modelling is used to eliminate the systematic error due to the camera/subject geometry, removing the dependence of luminescence intensity on animal position. Bioluminescence tomography (BLT) is then used to provide additional information about the depth and intensity of the source. A substantial limitation in the number of sources identified using BLI is also presented. It is shown that when a given source is at a significant depth, it can appear as multiple sources when imaged using BLI, while the use of BLT recovers the true number of sources present.

**Keywords:** Bioluminescence Imaging (BLI), Bioluminescence Tomography (BLT), Quantitative accuracy, Free Space Modelling, Image reconstruction

## 1. INTRODUCTION

Bioluminescence imaging (BLI) involves labelling a cell type of interest with a bioluminescent reporter gene (e.g. firefly luciferase, *fluc*) which emits light when a substrate (luciferin) is introduced. Images are taken of the light which reaches the surface of the animal, enabling information to be deduced about the location and dynamics of the labelled source cells.<sup>1,2</sup> The technique is widely used in pre-clinical cancer research to monitor the growth of tumours and the efficacy of novel treatments,<sup>1-3</sup> but can also be applied to a number of other applications, such as monitoring the migration and localisation of stem cells,<sup>4,5</sup> immune cells,<sup>6,7</sup> bacteria<sup>8,9</sup> and viruses.<sup>8,10,11</sup>

BLI provides major advantages over more traditionally used methods of monitoring tumour growth which involve injecting a group of animals with cancer cells at the same time-point. Animals are then sacrificed sequentially at different time-points following cell insertion to enable the size of the tumour to be determined. This method requires a large number of animals to be used in a single study and, due to the tumours being measured in different animals throughout the experiment, inter-animal variability of tumour growth is present. BLI dramatically reduces the number of animals which are required in a single experiment as tumour growth can be monitored longitudinally in each mouse over the entire experimental timescale. Images of each animal are taken at multiple time points after cell injection and the radiance is measured in a region of interest. This enables tumour growth to be monitored in each animal non-invasively, and negates the need to sacrifice animals at each time-point. Moreover, each animal is able to provide its own control measurements.

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However, in many of these pre-clinical studies little is known or investigated regarding the position, movement and underlying physiology of the animal, or the position of the bioluminescent source within the animal. All of these factors dramatically affect the quantitative accuracy of the obtained data.

This work presents preliminary data using an animal model to highlight some of the potential inaccuracies and drawbacks of using BLI. The dependence of measured BLI intensity on the position of the animal and depth of the source, and the uncertainty in observations made when analysing BLI images, will be identified. In each case, solutions to the problem are presented which demonstrate an increase in quantitative accuracy.

## 2. METHODS

A recently developed imaging system, with bioluminescence tomography (BLT), diffuse optical tomography (DOT) and surface capture capabilities (BLDOT, detailed in ref. [12]) is used to image three mouse cadavers with implanted artificial light sources (tritium-based, Trigelight Orange III; mb-microtec, Switzerland) inserted at different positions and depths within each animal.

The sources were imaged at 600 nm (using a band pass filter with a bandwidth of 22 nm) before insertion to obtain an accurate measure of the source intensity for quantitative comparison. The sources were inserted into the animals as follows:

Mouse 1 – subcutaneously dorsally, (Note: this animal was imaged in two different positions – referred to as mouse 1.1 and mouse 1.2);

Mouse 2 – at a depth of approximately 10 mm near the kidney;

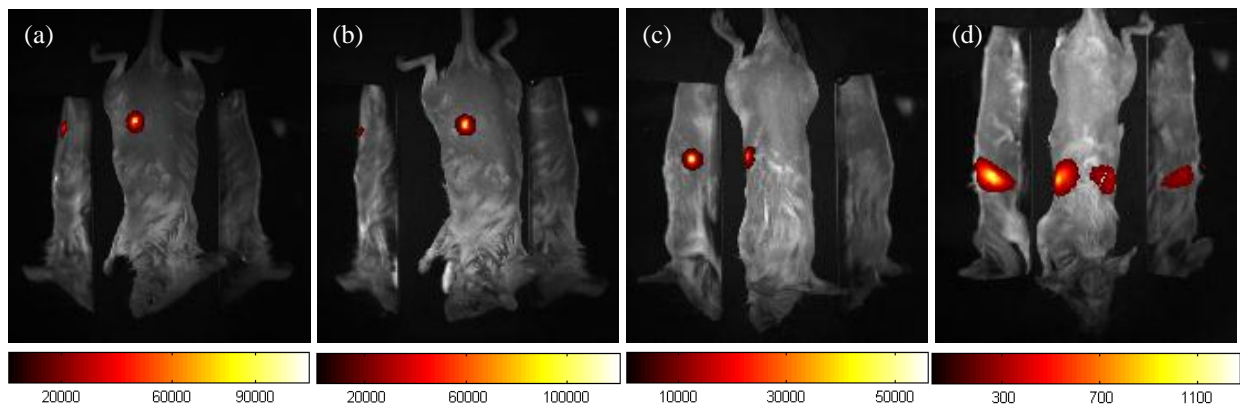
Mouse 3 – deep under the ribs, near the liver but within the chest.

Each animal was then imaged at 600, 623 and 643 nm (band pass filters with bandwidths 22, 32 and 34 nm respectively) to enable tomographic imaging, where performed.

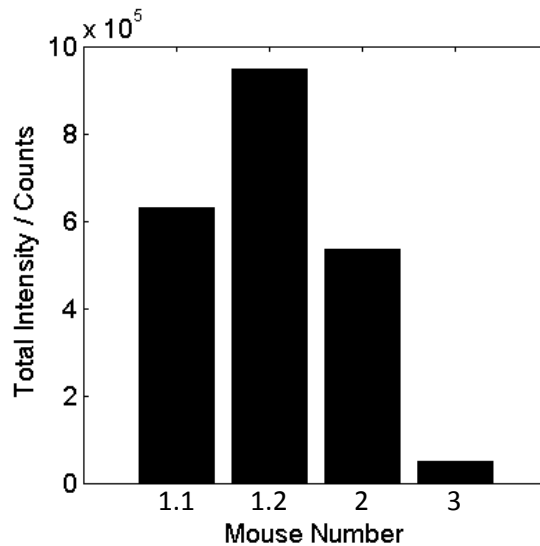
## 3. RESULTS AND DISCUSSION

### 3.1 Bioluminescence intensity is dependent on animal position

Bioluminescence images of the three animals in this study, overlaid onto bright images, are shown in Fig. 1. The change in position of Mouse 1 (Mouse 1.1 and 1.2, Fig. 1 a and b respectively) can be seen. Since the only parameter to alter between these two images is the position of the animal, it is expected that the measured intensity is equivalent in both cases. However, as evident in Fig. 2, the intensity measured for Mouse 1.2 is greater than that for Mouse 1.1.



**Figure 1** – Luminescence data of three mice: (a) Mouse 1.1, (b) Mouse 1.2, (c) Mouse 2 and (d) Mouse 3, each overlaid onto a bright image of the animal. Mirrors placed on either side of the animal (seen on either side of the direct camera view) expand the field of view of the system.



**Figure 2** – Total intensity of the top (direct) view of the bioluminescence images. The source was inserted subcutaneously in Mouse 1, and at increasing depths in Mice 2 and 3. A clear dependence of the intensity on the depth of the source is seen.

Changing the position of the animal with respect to the camera changes the perspective of the animal visible to the camera, and the observed appearance of the surface fluence is different. Assuming Lambertian emission from the imaged surface, the intensity of the light emitted from the surface in a particular direction is proportional to the cosine of the angle between the surface normal and the detection direction of the emitted light. Therefore a change in the position of the animal will change this angle, resulting in different measurements being made. This has been demonstrated previously using a homogeneous cylindrical phantom with an internal light source which had a variation in measured light intensity in excess of 50% when rotated through 90°. <sup>13,16</sup>

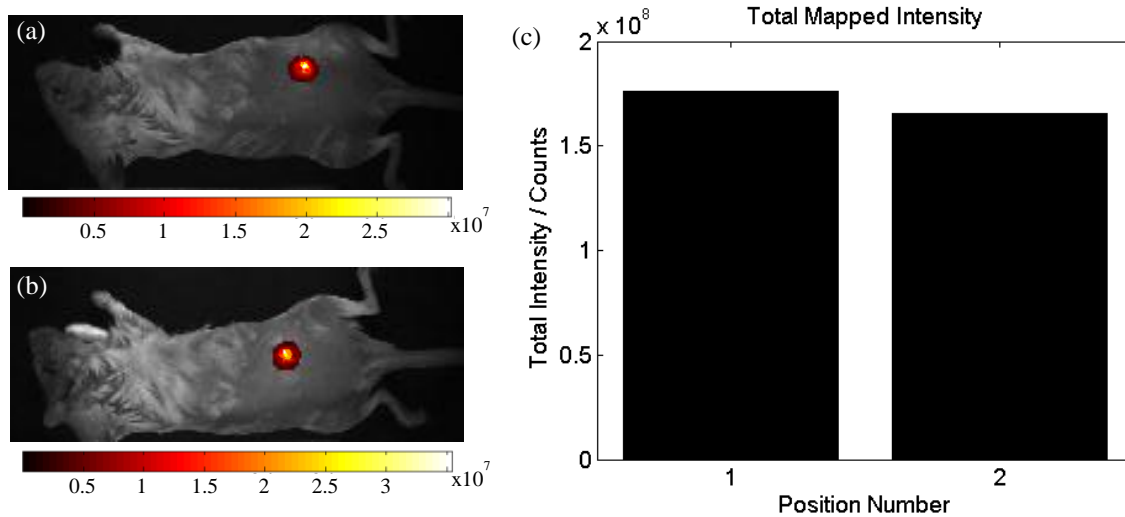
A recently developed Free Space Model <sup>13</sup> accounts for the propagation of light from the surface of the imaging subject to the CCD, calculating the contribution of each point on the surface of the imaging subject to the measurement at each pixel on the CCD. Inverting this relationship enables the measured bioluminescence to be mapped back onto the surface of the imaging subject, providing quantitatively accurate surface fluence measurements. In the case of the cylindrical phantom, the intensity variation after modelling was less than 20%. <sup>13,16</sup> The ability to gain quantitative information about the source in an animal regardless of its position is vital in order for accurate conclusions to be drawn in pre-clinical experiments.

Free Space Modelling was applied to data from Mouse 1 (Fig. 3 a and b). The total intensity of the mapped data (considering data above a threshold of 50% of the maximum intensity only, Fig. 3 c) is comparable for the two positions (the intensity of mapped data for Mouse 1.1 is 1.06× that of Mouse 1.2), showing that the inaccuracy in intensity observed in BLI images of the same animal is removed (the BLI intensity of Mouse 1.1 was two thirds of that of Mouse 1.2). Therefore it is demonstrated that the change in position of the animal can, and must, be accounted for. The resulting surface fluence measurements are independent of the position of the animal.

### 3.2 Bioluminescence intensity is dependent on source depth

Although the exact depth of the sources in this preliminary work is unknown, a clear dependence of measured intensity on source depth can still be observed. The total intensity for a region of interest across the top of the animal was calculated, demonstrating that the superficially inserted sources (Mouse 1.1 and 1.2) produce higher intensity measurements than the deeper sources (Mice 2 and 3), Fig. 2.

As all sources used in this work have similar intensities ( $5.25 \pm 0.011 \times 10^7$  counts s<sup>-1</sup>), all measured intensities should also be the same. This will enable quantitatively accurate data to be obtained and accurate conclusions to be drawn about the nature of the source. In order to achieve this, Free Space Modelling <sup>13</sup> (as described above) must be performed



**Figure 3** – Surface fluence data of (a) Mouse 1.1 and (b) Mouse 1.2 following Free Space modelling. Quantitative bioluminescence intensity information, independent of the position of the animal, has been produced (c).

to get accurate surface fluence, followed by BLT, which will provide three-dimensional volumetric information about the source.

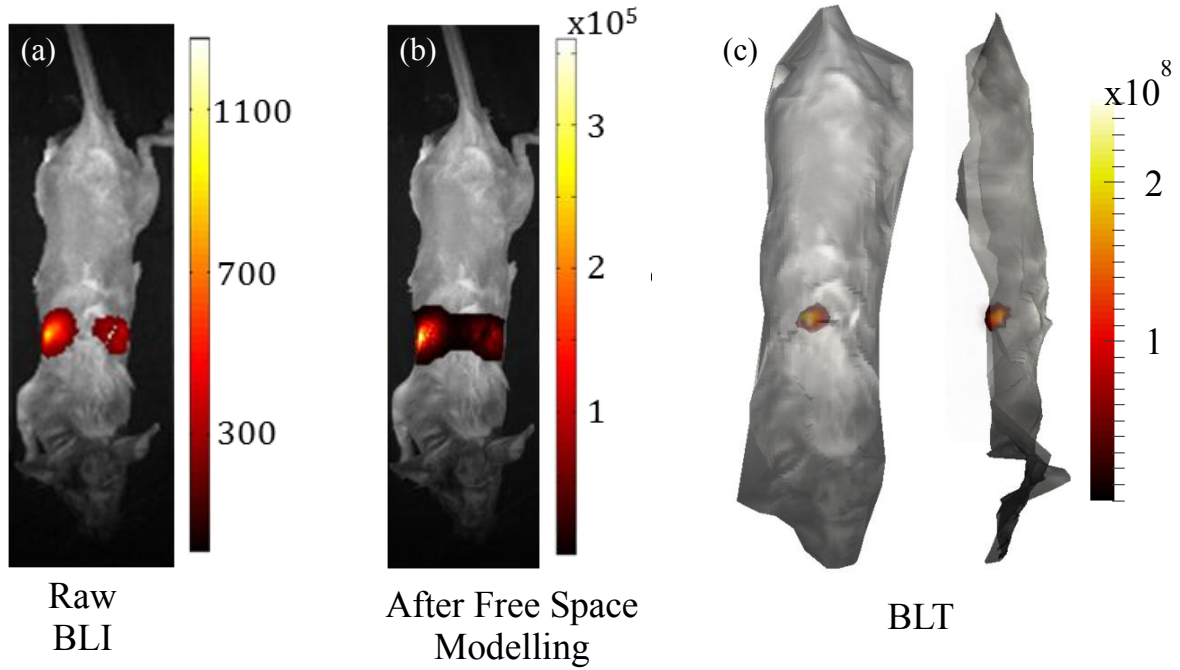
The ability to produce quantitatively accurate information about the source, in terms of location, intensity and size, is vital when performing pre-clinical studies if accurate information about tumour growth (for example) is to be obtained.

Work on more precise placement of the inserted source is on-going, and will lead to more in-depth quantitative analysis of the dependence of measured bioluminescence intensity on the depth of the source. But the data shown here demonstrates and highlights the necessity of tools and algorithms which are able to provide source information regardless of its location.

### 3.3 The number of sources present is unclear

The BLI images of Mouse 3, which had the deepest source, show two distinct regions of bioluminescence (Fig. 1 d and Fig. 4 a) suggesting that there are two sources present. However, in this case given the prior information about the location and number of sources (a single source inserted deep under the ribs but near the liver) such a conclusion is incorrect.

Free Space Modelling followed by BLT was performed on this data (Fig. 4). A Conjugate Gradient-based reconstruction algorithm using Compressive Sensing (CSCG, for more detail see ref. [14]) was used for tomographic reconstruction. The optical properties were assumed to be homogeneous (mouse ‘muscle’ optical properties were used, see ref. [15]). Fig. 4 c shows that a single source has been recovered, demonstrating that this workflow can successfully overcome difficulties in visually analysing BLI images, providing information about the true number of sources present. The intensity of the recovered source is approximately  $10^4$  counts larger than the actual source intensity, which can be due to incorrect assumptions regarding the bandwidth of the filters used. Specifically, although the data is collected using filters with bandwidths of  $\sim 20$  nm, the image reconstruction is making an assumption that the bandwidth is 1 nm. It is expected that a more accurate model, which accounts for the variation in filter bandwidth, will provide a more quantitatively accurate result.



**Figure 4** – Analysis workflow applied to mouse 3. The raw BLI image (a) displays two distinct regions of bioluminescence. The Free Space mapped data (b) corresponds to the raw BLI data, with corrected intensity. Bioluminescence tomography successfully recovers the true number of sources (c).

#### 4. CONCLUSIONS

A number of limitations to the quantitative accuracy of BLI have been identified and suggestions of methods to overcome these limitations have been made. Free Space Modelling has been shown to remove the dependence of luminescence intensity on position of the animal by modelling the light propagation through the free space between the imaging subject and the CCD and back again, mapping BLI data back onto the surface of the animal. The dependence of luminescence intensity on the depth of the source can be accounted for using Free Space Modelling to get quantitatively accurate surface fluence, followed by BLT to provide volumetric, depth-resolved information about the source. Data for Free Space Modelling and BLT of source depth is not shown in this manuscript as the data analysis is ongoing. For a sufficiently deep source BLI images may show two distinct regions of bioluminescence. Free Space Modelling followed by BLT was able to correctly recover a single source, removing any uncertainty in the number of sources present. This work has shown that these limitations must all be accounted for during pre-clinical imaging in order for quantitatively accurate source information to be obtained, independent of the position of the animal and the depth of the source, thus enabling accurate conclusions to be drawn about the nature and dynamics of the source.

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